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Regulation of Seed Protein Concentration in Soybean by Supra-Optimal Nitrogen Supply

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ABSTRACT

The physiological and biochemical basis for increased seed protein concentrations (SPC) observed in restriction-index, recurrent-selection breeding programs with soybean [*Glycine max* (L.) Merr.] are poorly understood. The hypothesis that soybean SPC is regulated by the supply of nitrogenous substrates available to the seed was evaluated. Effects of supra-optimal external N on seed storage protein accumulation, amino acid concentration and composition in leaves and seeds at R5, and levels of specific storage protein subunits were measured. Genotypes with different SPC (NC 107, normal; N87-984-16, intermediate; and NC 111, high) were grown in controlled-environment chambers and supplied with 30 m*M* N as NH_4NO_3 from V5 to maturity or from R5 to maturity. Control plants received 10 m*M* N throughout the growth cycle. Relative to control, supra-optimal N increased SPC of NC 107 and N87-984-16 by an average of 28%. Greater enhancement of protein accumulation than of dry matter accumulation in the seed resulted in SPCs of 460 to 470 g kg^{-1}, which are appreciably greater than concentrations observed for these cultivars grown in the field. Supra-optimal N also increased SPC of the high protein line (NC 111) by 15%, but this increase resulted entirely from a decrease in yield. Supra-optimal N supplied to NC 107 and N87-984-16 from V5 until R5 increased total free amino acid concentrations in seeds and leaves at R5 by an average of 21 and 46%, respectively. Enhanced accumulation of the β subunit of β conglycinin which does not contain methionine and cysteine accounted for the increase in SPC. While enhanced N availability increased the SPC of a normal protein line into the high range, availability of sulfur amino acids in the developing seed determined which storage protein subunits were synthesized from the extra N.

While considerable variation in SPC exists within soybean germplasm (350–500 g kg^{-1} dry weight), it has been difficult to enhance this trait in soybean seed without lowering seed yield and oil concentration (Brim and Burton, 1979; Miller and Fehr, 1979). These negative impacts on yield and constituent value remain significant barriers to development of high-protein commercial cultivars.

Soybean SPC is inherited as a quantitative trait (Burton, 1987) and influenced by environmental effects (Burton, 1988). Generally the trait is much less influenced by the genotype of the embryo than by the genotype on which the seeds develop (Singh and Hadley, 1968). This suggests that whole plant processes such as N acquisition, translocation, and mobilization of C and N are important in the determination of seed protein concentration.

Brim and Burton (1979) used recurrent selection to increase SPCs in two populations (IA and IIA). Carter et al. (1982) examined relationships between N accumulation and distribution and SPC in high and low seed germplasm from both populations. They observed that high protein germplasm from population IA accumulated more total N before reproductive development than high seed protein germplasm in population IIA. Selected high and low seed protein lines derived from Cycle 0 and advanced cycles of selection for both populations were evaluated for vegetative N accumulation prior to reproductive growth and vegetative N mobilization to seed during reproductive growth (Burton et al., 1995). The authors concluded that the high protein concentration trait resulted neither from greater vegetative N accumulation before R5 nor from greater N mobilization from vegetative tissue to developing seed since normal and high seed protein lines did not differ significantly in these attributes. Thus, measurements of whole plant N accumulation and vegetative N mobilization have not provided a clear understanding of how SPC in soybean is regulated.

Saravitz and Raper (1995) evaluated the C and N requirements of 'Ransom' soybean embryos in in vitro culture from 17 to 41 d after flowering (DAF). When 150 m*M* sucrose was used as the C source, the protein concentration in the embryo increased from 150 to 690 g kg^{-1} dry weight as the glutamine concentration was increased from 0.6 to 120 m*M*. The protein concentration at the highest glutamine concentration (690 g kg^{-1}

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Abbreviations: DAF, days after flowering; DAT, days after transplant; SPC, seed protein concentration; YFEL, youngest fully expanded leaves.

Table 1. Daily N application for each treatment at different stages of growth.

Growth interval	Treatments		
	Control	30 m*M* N after V5	30 m*M* N after R5
DAT†	mmol N d^{-1}		
4–20 (V5)‡	2.5	2.5	2.5
20–76 (R5)§	5.0	15.0	5.0
76–maturity¶	10.0	30.0	30.0

† Days after transplanting.
‡ Vegetative stage when plants have six nodes with leaves with unfolded leaflets.
§ Beginning seed fill stage when plants have pods at uppermost four nodes on main stem with seeds 3 mm in length.
¶ After R5 stage, 0.5 L of nutrient solution was applied after both morning and afternoon flushes with deionized water, as plants in the control treatment (10 m*M* N) showed a moderate N deficiency with a single daily application.

dry wt.) is much higher than that (500 or 550 g kg^{-1} dry wt.) considered to be high for soybean seeds produced on an intact plant. When grown under field conditions, the Ransom cultivar has SPC of 380 to 400 g kg^{-1} dry weight. In this system, the embryos developing in high glutamine medium could be considered to have an unlimited supply of N available to support protein biosynthesis. The dry mass of embryos also doubled as the protein concentration increased with increased glutamine supply (Saravitz and Raper, 1995). Therefore, restriction of growth by glutamine did not cause the increase in protein concentration. These results imply that soybean seeds of this normal seed protein cultivar have intrinsic biochemical capacity to synthesize high protein concentrations and that N available to the developing seed may regulate SPC.

The objective of our research was to test the hypothesis that high SPC trait is regulated by the supply of nitrogenous substrates to the developing seeds by measuring the effects of supra-optimal external N supply on seed protein formation and associated physiological and biochemical processes in genotypes that exhibit normal and high SPC. If N supplied by the plant limits SPC, supply of supra-optimal N to normal protein lines is predicted to increase SPC.

MATERIALS AND METHODS

Plant Culture

Normal (NC 107) and high (NC 111) protein lines derived from recurrent selection populations (Carter et al., 1986) and N 87-984-16, an intermediate protein line derived from restriction-index recurrent selection, were used for this study (Burton, 1988). Seeds were pregerminated in germination paper saturated with 0.5 m*M* $CaSO_4$ at 28°C and 95% relative humidity for 72 h before transplanting into 25.4-cm pots filled with coarse perlite. Two to three seedlings were transplanted in each pot and thinned to one plant per pot at 14 d after transplanting (DAT). Plants were grown in two controlled-environment chambers with two replications in each chamber in the Southeastern Plant Environment Laboratories at North Carolina State University. The photosynthetic photon flux density of ≈850 to 900 μmol m^{-2} s^{-1} between wavelengths of 400 to 700 nm was provided by a mixture of super metal halide and incandescent lamps (Downs and Thomas, 1990). Plants were grown with a 14-h photoperiod for 36 DAT (until V6 or V7) and then switched to a 12-h photoperiod for the remainder of the experiment to initiate reproductive development. Day and night temperature of 26 and 22°C, respectively, were used throughout the growth cycle.

Nutritional Treatments

With the exception of inorganic N, the composition of nutrient solutions was the same as that previously described by McClure and Israel (1979). From the day of transplanting until 3 DAT, noninoculated plants were supplied only 0.25 L of deionized water. From 4 DAT until the N treatments were imposed, pots were flushed with 1.0 L of deionized water at 0800 and 1300 h and supplied with nutrient solution as shown in Table 1.

Since the pH of the root zone is altered by the form of N being absorbed, the pH of the bulk solution within the solid substrate was measured twice weekly by collecting the first 0.5 L of solution that drained from the bottom of the pots during flushing with deionized water in the morning. The ratio of NH_4 and NO_3 was adjusted to maintain the pH of the root zone within the range of 5 to 7. When the desired NH_4NO_3 ratio was <1.0, KNO_3 was substituted for the appropriate amount of NH_4NO_3.

Sampling Procedures

Four replicates of the control and N-treated plants were harvested at R5 and maturity. Plants were separated into leaves, stem, roots, seeds, and pod walls and oven-dried at 60°C for 72 h. Dry mass and N concentration of plant parts and whole plants were measured.

Tissue Nitrogen and Sulfur Analysis

Samples (50–100 mg) of each plant part were digested by a Kjeldahl procedure using a zirconium copper catalyst (Glowa, 1974) and a salicylic acid predigestion step to convert NO_3 to NH_4 (Nelson and Sommers, 1973). Digests were alkalinized with 10 *M* NaOH, and NH_3 was steam-distilled into 0.32 *M* boric acid and quantified by titration with 0.01 *M* potassium biiodate. Nitrogen percentage in seed was converted to protein percentage by multiplying by the conversion factor, 6.25. Sulfur concentration in seed was determined by inductively coupled plasma emission spectrometry after sample digestion in concentrated nitric acid (Novozamsky et al., 1986).

Amino Acid Analysis

At R5, leaves, seeds, and pod wall were subsampled for amino acid analysis. Six leaf-discs (total area of 2.65 cm^2) were collected from the youngest fully expanded leaves (YFEL). Five-hundred milligrams of seeds and pod walls were subsampled and cut into small pieces. These plant parts were frozen in liquid N and placed in a freezer at −80°C until the analysis was performed.

Approximately 0.5, 0.5, and 0.05 g fresh weight of seeds, pod wall, and leaf were homogenized in 13.7 *M* ethanol using the Brinkman Polytron (Brinkman Instruments, Westbury, NY) for extraction of soluble N. Homogenates were centrifuged at 1240 *g* in a swinging bucket centrifuge (Model CR411, Jouan Inc., East Winchester, VA). The pellets were extracted two more times by suspending in 13.7 *M* ethanol followed by centrifugation. Three ethanolic supernatant fractions containing soluble N were combined. Duplicate aliquots of the combined ethanolic extracts of each sample were used for determination of free amino acid composition. Free amino acids in soluble N fractions were derivatized with phenylisothiocyanate and separated and quantified using reversed-phase

high performance liquid chromatography (Heinrikson and Meredith, 1984).

Total Seed Storage Protein Profiles

Flowers were tagged on the day of opening, and then pods were collected at 15, 30, 45, and 60 d after tagging. Seeds were separated from pod wall, frozen in liquid N, and stored in a freezer at −80°C until the analysis could be performed. Seeds were extracted by homogenizing in 1:10 (w/v) 0.03 *M* Tris-HCl buffer, pH 8.0, and 7.7 m*M* NaN_3, using a Brinkman Polytron. Homogenates were centrifuged at 10 000 *g* using the RC5C centrifuge (Sorvall Instruments, Dupont, Wilmington, DE). The soluble protein concentration in the extracts was then determined by the method of Bradford (1976) with bovine plasma gamma globulin (Bio-Rad Inc., Richmond, CA) as the standard. Seed proteins were denatured in 0.03 *M* Tris-HCl buffer, pH 8.0, 69 mM SDS, and 0.28 *M* β mercaptoethanol in a boiling water bath for 10 min.

Electrophoresis was carried out using the buffer system described by Chua (1980) with 12.5 to 20% linear gradient polyacrylamide gel of 1.5-mm thickness for 18 h at 6 mA. Gels were stained with Coomassie brilliant blue R-250 as described in Harlow and Lane (1988). Total seed protein profiles were quantified with a Molecular Dynamics Personal Densitometer SI equipped with a HeNe laser light source and ImageQuant software for volume integration of total optical density of entire protein bands (Molecular Dynamics, Sunnyvale, CA). The amount of protein on gels was adjusted to ensure that the most intense bands were within the linear response range of the detector. Storage protein subunits were identified by comparison with molecular weight standards run in outside lanes on each gel. The relative amount of each storage protein subunit was expressed as a percentage of total storage protein in the gel lane.

Experimental Design and Statistical Analysis

For growth parameters and N analysis, the treatment structure was defined based on two levels of N, 10 m*M* N (control) and 30 m*M* N applied during two different growth stages, V5 to maturity and R5 to maturity. Plant samples were harvested at two different stages, R5 and maturity. This resulted in the following treatment combinations: (i) 10 m*M* N from 4 DAT to maturity (control), (ii) 10 m*M* N from 4 DAT to V5 and 30 mM N from V5 to maturity, and (iii) 10 m*M* N from 4 DAT to R5 and 30 m*M* N from R5 to maturity.

These treatments were arranged in a randomized complete block design with four replications (two replications in each chamber). The statistical analysis revealed no significant chamber effect on any parameters; thus, a randomized complete block design with four replications is appropriate for this experiment. The data for all measured parameters were analyzed using the ANOVA procedure of the Statistical Analysis System (Goodnight, 1982). If treatment effects were significant at the 0.05 probability level, $LSD_{0.05}$ values were calculated for comparison of means.

A randomized complete block design with three replications was used for analysis of free amino acid composition data. Treatments consisted of a 3 by 2 factorial with three soybean varieties and two N levels. The data for the amino acid concentration and composition were analyzed using the ANOVA procedure of the Statistical Analysis System (Goodnight, 1982). If treatment effects were significant at the 0.05 probability level, $LSD_{0.05}$ values were calculated for comparison of means.

For total seed storage protein profile measurements, two replicates of treatment combinations as described above were used for 30, 45, and 60 DAF. Because the plants reached R5 stage after 15 DAF, a 3 by 2 factorial of three varieties and two N levels was applied at 15 DAF. Data are reported as the percentage of each individual storage protein band relative to the total amount of storage protein on the gel at each sampling date.

RESULTS

Nitrogen Assimilation

Cultivars NC 107 and N87-984-16 had similar seed yields and whole plant dry weights, but both had significantly higher seed yields than NC 111 (Table 2). Relative to the control, 30 m*M* N supplied from V5 and R5 to maturity increased seed yield of NC 107 and N 87-984-16 by an average of 45 and 55%, respectively, and decreased seed yield for NC 111 by 38 and 30%, respectively (Table 2). The 30 m*M* N treatments had no significant effect

Table 2. Effect of 30 m*M* external N supplied after V5 and R5 on whole plant dry matter accumulation and seed yields. Values for individual treatments are means of four replicate plants.

Genotype	Time of N application	N concentration	Seed yield	Whole plant dry matter
		mM	g plant^{-1}	
NC 107	V5	10	83	244
NC 107	V5	30	136	362
NC 107	R5	30	106	311
		Genotypic means	108	306
N87-984-16	V5	10	75	246
N87-984-16	V5	30	123	428
N87-984-16	R5	30	108	365
		Genotypic means	102	346
NC 111	V5	10	61	206
NC 111	V5	30	38	167
NC 111	R5	30	43	185
		Genotypic means	47	186
			N treatment means	
		10 mM-V5	73	232
		30 mM-V5	96	319
		30 mM-R5	86	287
LSD0.05				
Genotypes			21	64
N treatments			21	64

on seed numbers (data not shown) for these varieties; thus 30 m*M* N treatments increased seed yield primarily by increasing seed size. We have no explanation for the negative effect of 30 m*M* N treatment on seed yield of NC 111.

Growth at the intensity (850 μeinsteins m^{-2} s^{-1}) and duration of light (12 h d^{-1}) was so rapid that plants of NC 107 and N87-984-16 in the 10 m*M* N treatment showed moderate symptoms of N deficiency (i.e., light green leaves) after R5 stage. This resulted in lower SPC (Table 3) in NC 107 (345 g kg^{-1} dry wt.) and N87-984-16 (387 g kg^{-1} dry wt.) than has been observed for these two cultivars grown in the field and outdoor pot culture (SPC of 400 g kg^{-1} dry wt. for NC 107 and 450 g kg^{-1} dry wt. for N87-984-16). However, supply of 30 m*M* N from V5 and R5 to maturity increased total seed N per plant by 124 and 71% in NC 107 and 106 and 73% in N87-984-16 compared with the 10 m*M* N control (Table 4). The greater increase in total seed N accumulation in response to 30 m*M* N than in seed dry matter accumulation (Table 2) resulted in highly significant increases in SPC of 37 and 35% for NC 107 and 25 and 20% for N87-984-16 when 30 m*M* N was supplied after V5 and R5, respectively (Table 3). Supra-optimal N also increased SPC of the high protein genotype (NC 111), but a decrease in seed yield rather than an increase in seed N accumulation accounted for its increased SPC (Tables 2 and 4). For all cultivars, the Kjeldahl protein concentration of mature seed after extraction with 13.7 *M* ethanol increased to the same extent as total Kjeldahl protein concentration in response to supra-optimal N (Table 3). Since ethanol extraction removes soluble nonprotein forms of N, this result indicates that supra-optimal N enhanced protein accumulation rather than soluble N accumulation in seed.

Free Amino Acid Concentration

Since glutamine is the predominate amino acid translocated from leaves to seed of soybean and is readily metabolized to other amino acids required for protein biosynthesis (Rainbird et al., 1984), the influence of N supply on the glutamate plus glutamine (Glx) fraction of the free amino acid pool in YFEL and seed at R5 was of particular interest. Supply of 30 m*M* N after V5 had no significant effect on the concentration of Glx and aspartate plus asparagine (Asx) in seed when compared with the control for all three varieties (Table 5), but significantly increased the total free amino acid concentration in seed of NC 107 and N87-984-16 (18 and 24%, respectively). Supra-optimal N significantly increased Glx, Asx, and total free amino acid concentration in YFEL of NC 107 and N87-984-16 by an average of 194, 123, and 46%, respectively, when compared with the 10 m*M* N control. In contrast, supra-optimal N significantly decreased seed total free amino acid concentration of NC 111 by 52% and significantly increased the concentration of total free amino acid in YFEL by 38%. While the concentration was rather high in YFEL of all three varieties, it did not increase in response to increased N supply.

Seed Storage Protein Profiles

Total seed storage protein profiles were measured by SDS gel electrophoresis of seed protein extracts at 15, 30, 45, and 60 DAF to determine whether the increases in protein concentration were due to enhancement of specific seed storage protein subunits or to enhancement of all storage protein subunits. In general, α and α′ subunits of 7S protein appeared first (15 DAF), followed by the β subunit of 7S protein and acidic and basic subunits of 11S proteins (data not shown). Consistent with results of several studies (Goldberg et al., 1981; Meinke et al., 1981; Kondo et al., 1986), accumulation of all seed storage protein subunits was rapid from 15 to 45 DAF (data not shown). The percentage of 11S protein subunits increased from 15 to 60 DAF, while the percentage of 7S protein subunits decreased during this time period for all varieties (data not shown). Application of 30 m*M* N after V5 and R5 significantly increased the relative amounts of the β subunit of the 7S

Table 3. Comparison of measurements of protein concentrations in soybean seed by different methods as influenced by genotype and N supply. Values for individual treatments are the means for four replicate plants.

Genotype	N conc.	Time N applied	Total Kjeldahl protein	Kjeldahl protein after 13.7 *M* ethanol extraction†
	m*M*		g kg^{-1} dry weight	
NC 107	10	V5	345	334
	30	V5	473	460
	30	R5	466	453
N87-984-16	10	V5	387	375
	30	V5	483	468
	30	R5	464	451
NC 111	10	V5	472	457
	30	V5	550	522
	30	R5	524	507
LSD0.05 Genotype × N treatment			25	26

† Seed protein concentration corrected for ethanol-soluble N forms.

Table 4. Effect of 30 m*M* external N supplied after V5 and R5 on N accumulation in whole plant and in seed. Values for individual treatments are means for four replicate plants.

Genotype	N conc.	Time of application	Total seed N	Whole plant N
	m*M*		g $plant^{-1}$	
NC 107	10	V5	4.6	6.5
NC 107	30	V5	10.2	14.9
NC 107	30	R5	7.8	11.8
	Genotypic means		7.5	11.1
87-984-16	10	V5	4.6	6.7
87-984-16	30	V5	9.5	15.8
87-984-16	30	R5	8.0	13.3
	Genotypic means		7.4	11.9
NC 111	10	V5	4.6	6.9
NC 111	30	V5	2.6	7.1
NC 111	30	R5	3.6	7.8
	Genotypic means		3.6	7.3
			N treatment means	
	10 mM N-V5		4.6	6.7
	30 mM N-V5		7.4	12.6
	30 mM N-R5		6.5	11.0
LSD0.05				
Genotypes			1.6	2.1
N treatments			1.6	2.1

Table 5. Effect of 30 m*M* N supplied after V4 and R5 on amino acid concentrations in leaves and seeds. Values for individual treatments are means for three replicate plants.

Genotype	N conc.	Plant part	Amino acid					
			Asx‡	Glx‡	His	Phe	Other§	Total
	m*M*		mmol kg^{-1} fresh weight					
NC 107	10	Seed	13	62	30	19	85	209
	30		20	58	57	17	94	246
87-984-16	10		16	59	50	16	77	218
	30		18	75	58	17	103	271
NC 111	10		26	69	66	17	100	277
	30		12	22	55	29	66	184
Significance level								
Genotype			NS	NS	NS	NS	NS	NS
N level			NS	NS	NS	NS	NS	NS
Geno. × N level			0.05	0.05	NS	NS	0.05	0.05
NC 107	10	YFEL†	5	5	Tr	53	53	116
	30		13	16	4	50	94	178
87-984-16	10		8	5	Tr	53	61	127
	30		15	14	1	51	95	177
NC 111	10		16	14	4	54	92	180
	30		12	15	7	74	139	247
Significance level								
Genotype			0.05	0.01	0.01	NS	0.05	0.05
N level			0.05	0.01	0.01	NS	0.01	0.01
Geno. × N level			0.05	0.01	NS	NS	NS	NS

† Youngest fully expanded leaves.
‡ Asx = aspartic acid + asparagine and Gsx = glutamic acid + glutamine.
§ Other = sum of Ser, Gly, Arg, Thr, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, and Lys.

protein fraction and significantly decreased the relative amounts of the basic subunit of the 11S protein fraction in seed at 60 DAF, particularly for NC 107 (Table 6). The reciprocal changes in the relative amounts of β subunit and basic subunit in response to application of 30 m*M* N caused decreases in the 11S/7S ratio. The relative amounts of other storage protein subunits were not significantly altered by addition of 30 m*M* N.

The reciprocal changes in relative amounts of 7S and 11S protein subunits in seed at 60 DAF in response to supra-optimal N were most pronounced for the normal protein genotype (NC 107) and intermediate for the intermediate protein genotype N87-984-16 (Tables 6 and 7). Supra-optimal N had no appreciable influence on seed storage protein composition of the high protein genotype NC 111.

Sulfur Status of Seed

Since a positive relationship between the proportion of β subunit in seed storage protein and the N/S ratio of seed has been noted (Sexton et al., 1998), the effect of supra-optimal N on S status of seed was evaluated (Table 8). Supra-optimal N had no significant effect on the S concentrations in seed and a highly significant effect on the N/S ratio in the seed (Table 8). The significant increase in the N/S ratio in response to supra-optimal N

Table 6. Effect of 30 m*M* N supplied after V5 and R5 on relative amounts of seed storage protein subunits at maturity (60 d after flowering). Values for individual treatments are the means of two replicate plants.

Genotype	N conc.	Time N applied	Protein subunit						
			7S fraction			11S fraction			
			α′	α	β	A3	A5	Acidic	Basic
	m*M*		% of total storage protein						
NC 107	10	V5	3.2	11.1	6.7	5.5	1.8	35.2	36.7
	30	V5	4.4	13.7	15.1	5.7	0.5	34.0	26.9
	30	R5	5.5	13.0	14.5	4.6	0.5	38.2	23.9
	Genotypic means		4.3	12.6	12.1	5.3	0.9	35.8	29.1
87-984-16	10	V5	5.1	17.5	15.9	6.2	0.4	30.6	24.6
	30	V5	6.9	14.9	21.9	6.6	0.3	29.5	19.9
	30	R5	8.6	16.9	17.4	6.0	0.3	35.0	16.0
	Genotypic means		6.9	16.4	18.4	6.2	0.3	31.7	20.2
NC 111	10	V5	8.5	9.8	9.5	6.4	0.7	36.2	28.9
	30	V5	5.9	8.9	10.3	4.4	0.6	36.3	33.7
	30	R5	5.5	9.8	9.7	5.7	0.7	41.6	27.2
	Genotypic means		6.6	9.5	9.8	5.5	0.7	38.0	29.9
			N treatment means						
	10 mM-V5		5.6	12.8	10.7	6.0	1.0	34.0	30.0
	30 mM-V5		5.7	12.5	15.8	5.6	0.5	33.3	26.8
	30 mM-R5		6.5	13.2	13.9	5.4	0.5	38.2	22.4
LSD0.05									
Genotypes			2.3	3.8	4.1	NS	0.6	4.4	3.8
N treatments			NS	NS	4.1	NS	NS	4.4	3.8

Table 7. Effect of 30 m*M* N supplied after V5 and R5 on relative amounts of 11S and 7S storage protein fractions. Values for individual treatments are the means of two replicate plants.

Genotype	N conc.	Time N applied	Storage protein fraction 7S	11S	11S/7S
	m*M*		% of total storage protein		ratio
NC 107	10	V5	21	79	3.8
	30	V5	33	67	2.0
	30	R5	33	67	2.0
	Genotypic means		29	71	2.5
87-984-16	10	V5	38	62	1.6
	30	V5	44	56	1.3
	30	R5	43	57	1.3
	Genotypic means		42	58	1.4
NC 111	10	V5	28	72	2.6
	30	V5	25	75	3.0
	30	R5	25	75	3.0
	Genotypic means		26	74	2.9
			N treatment means		
	10 mM N-V5		29	71	2.5
	30 mM N-V5		34	66	2.0
	30 mM N-R5		34	66	2.0
LSD0.05					
Genotypes			4.3	4.4	NS
N treatments			4.3	4.4	NS

was due entirely to the increase in seed N concentration (Table 8).

DISCUSSION

Nutritional Regulation of Protein Concentration

Since the 30 m*M* N treatment increased SPC of NC 107 and N87-984-16 to levels (460–470 g kg^{-1} dry wt.) appreciably higher than those reported for these varieties grown in the field and outdoor pot culture (400–440 g kg^{-1}; Burton et al., 1995), this treatment apparently exceeded the external N level required for optimal seed yield. Similar increases in SPC as measured by Kjeldahl N analysis before and after ethanol extraction in response to supra-optimal N were observed for all cultivars (Table 3). Thus, increased N concentration in seed of all cultivars was primarily the result of protein accumulation rather than soluble N accumulation. These results indicate that SPC of normal seed protein lines can be increased into the high SPC range by increasing the external N concentration to supra-optimal levels during reproductive growth. This suggests that developing seed of this normal seed protein line have the intrinsic biochemical capacity to synthesize high protein concentrations when adequate substrate is available.

Since leaves contribute most of the nitrogenous substrates to developing seed (Rainbird et al., 1984), the increase in total amino acid concentration in leaves (Table 5), particularly Glx and Asx, may have contributed to increased SPC in NC 107 and N87-984-16 in response to 30 m*M* N (Table 5). Barneix and Guitman (1993) also concluded that protein biosynthesis in wheat (*Triticum aestivum* L.) grain is substrate-limited by the amino acid pool in the leaves and that increasing the amino acid pool in the leaves could enhance export of amino acids to the grain.

In vitro seed culture experiments have led to different conclusions about the regulation of SPC. Hayati et al. (1996) evaluated the C and N requirements of embryos of soybean cultivars with different SPC. When sucrose was maintained at 200 m*M* and the N concentration in the media varied from zero to 270 m*M* as a 6.25:1 molar ratio of asparagine to methionine, the cotyledon protein concentration of the high protein genotype was higher than that of a normal protein genotype at all levels of N. At the highest external N level (270 m*M*) protein concentrations were 457 and 375 g kg^{-1} dry weight for the high and normal protein genotypes, respectively. These results were interpreted to support the conclusion that processes within the cotyledons of developing seeds may regulate genetic differences in SPC. Saravitz and Raper (1995) evaluated the C and N requirements of Ransom soybean embryos in in vitro culture from 17 to 41 DAF. When 150 m*M* sucrose was used as the C source, the protein concentration in the embryo increased from 150 to 690 g kg^{-1} dry weight as the glutamine concentration was increased from 0.6 to 120 m*M*.

Table 8. Effect of 30 m*M* N after V5 and R5 on the N and S composition of mature seed. Values represent the mean of four replicate plants.

Genotype	Time of N application	N concentration	Seed N concentration	Seed S concentration	Seed N/S ratio
		m*M*	g kg^{-1} dry wt.		
NC 107	V5	10	55.2	3.9	14.0
	V5	30	75.7	3.8	19.8
	R5	30	74.6	4.1	18.1
	Genotypic means		68.5	3.9	17.3
N87-984-16	V5	10	61.9	4.2	14.7
	V5	30	77.3	4.4	17.6
	R5	30	74.2	4.2	17.7
	Genotypic means		71.1	4.3	16.7
NC 111	V5	10	75.5	3.7	20.4
	V5	30	88.0	3.6	23.8
	R5	30	83.8	3.9	21.5
	Genotypic means		82.4	3.7	21.9
				N treatment means	
	10 mM-V5		64.2	4.1	16.5
	30 mM-V5		77.5	4.0	19.1
	30 mM-R5		80.3	3.9	20.5
LSD0.05					
Genotypes			2.3	0.3	1.2
N treatments			2.3	NS	1.2

The protein concentration at the highest glutamine concentration (690 g kg^{-1} dry weight) is much higher than the protein concentration (500 or 550 g kg^{-1} dry wt.) that is considered high for soybean seeds produced on an intact plant. When grown under field conditions, the Ransom cultivar has SPCs of 380 to 400 g kg^{-1} dry weight. In this system, the embryos developing in high glutamine medium could be considered to have an unlimited supply of N available to support protein biosynthesis. These results imply that soybean seeds of this normal seed protein cultivar have intrinsic biochemical capacity to synthesize high protein concentrations and that N available to the developing seed may regulate SPC.

The contrasting observations and conclusions of Hayati et al. (1996) and Saravitz and Raper (1995) may be related to the use of different sources of N in the culture media (i.e., asparagine and glutamine, respectively). Thompson et al. (1977) reported that while glutamine and asparagine were equally effective in promoting dry matter accumulation in cultured seed, glutamine promoted greater protein accumulation than asparagine. Wettlaufer and Obendorf (1991) observed that glutamine was superior to asparagine in supporting growth of seeds that weighed 50 to 70 mg fresh weight at the time of culture initiation when the media contained either 0.1 or 1.2 m*M* P. Seeds of the normal protein cultivar Ransom accumulated 690 g protein kg^{-1} dry weight when supplied 120 m*M* glutamine (Saravitz and Raper, 1995), whereas, the high protein cultivar BARC-8 accumulated only 457 g protein kg^{-1} dry weight of seed when supplied 270 m*M* asparagine (Hayati et al., 1996). These considerations indicate that SPC of the cultivars in the study of Hayati et al. (1996) was probably limited by the capacity of the developing seed to absorb and metabolize asparagine. Since glutamine is the predominate form of N in seed coat exudate (Rainbird et al., 1984), the results of Saravitz and Raper (1996) are more applicable to whole soybean plants and thus are consistent with the conclusion from the present study with intact plants that SPC of a "normal" soybean line (NC 107) is regulated by N availability to the developing seed.

Nutritional Regulation of Protein Quality

The increase in SPC for NC 107 and N87-984-16 in response to supra-optimal N (Table 4) was associated with increased relative amounts of the β subunit of 7S protein and decreased amounts of the basic polypeptide of 11S protein (Tables 6 and 7). Ohtake et al. (1997) reported that seeds of N deficient nonnodulated soybean plants lacked measurable β subunit and that seeds of nodulated soybeans exhibited respectable levels of β subunit, which increased when additional inorganic N was supplied. High levels of the β subunit mRNA were measured in seed of the nodulated line and none was measured in seed of the N deficient nonnodulated line.

Sulfur nutrition has also been shown to have a profound influence on accumulation of the β subunit of β conglycinin (7S protein). For example, when S nutrition of cultured soybean cotyledons was enhanced by addition of methionine to the culture medium the β subunit did not accumulate and its mRNA was not present (Holowach et al., 1984; Thompson et al., 1984). These results indicate that methionine down regulates transcription of the gene coding for the β subunit of β conglycinin. In studies with intact plants, Sexton et al. (1998) showed that increasing S supply from deficient to sufficient levels decreased the seed N/S ratio from 40:1 to 15:1 and the β subunit level in soybean seed from 40% of total storage protein to 10%. In our study, the seed N/S ratio increased from 14:1 to 24:1 as a result of increased N concentration in the supra-optimal N treatments (Table 8). The increased N/S ratio was due entirely to an increase in N concentration as S concentration was not changed by the supra-optimal N treatments (Table 8). The β subunit composition ranged from 7 to 21% of the seed storage protein across all combinations of genotype and N treatment (Table 6).

Results of this study coupled with those of Ohtake et al. (1997) and Sexton et al. (1998) support the concept that interactions between N and S metabolism influence seed storage protein composition in soybean. The β subunit of β conglycinin does not contain methionine and cysteine (Tierney et al., 1987). Under N deficiency that resulted from growing nonnodulated soybean in fields without N fertilization, no β subunit protein or transcript was measured (Ohtake et al., 1997). At the low protein synthesis rates in seed of N deficient plants, sulfur amino acid synthesis was apparently sufficient to support synthesis of subunits that contain sulfur amino acids and to prevent accumulation of the β subunit by suppressing transcription of its gene. When S deficiency of soybean plants with an adequate supply of N was relieved, the β subunit composition decreased from 40 to 10% of the total storage protein (Sexton et al., 1998). The β subunit composition was high in S-deficient seed because availability of sulfur amino acids limited the synthesis of subunits that contain these amino acids. The failure of β subunits to decrease to zero at high S levels and adequate N indicates that at the higher rates of protein formation sulfur amino acid levels were not sufficient to sustain synthesis of subunits that contain methionine and cysteine and to suppress expression of the gene that codes for the β subunit. In the present study, a significant portion of the increase in seed storage protein concentration in NC 107 in response to supra-optimal N was due to an increase in the level of β subunit (Table 6). This indicates that supply of sulfur amino acids was not sufficient to sustain synthesis of subunits (11S) which contain these amino acids and that up regulation of β subunit genes permitted assimilation of additional N into storage protein of lower nutritional quality.

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